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Abstract: Retinoid treatment is suggested to promote development of inflammatory bowel disease, although preclinical studies are not supportive. We evaluated the effect of retinoids on cytokine response in in vitro-differentiated human dendritic cells (ivDCs) and macrophages (ivMACs) derived from healthy human donors and in cultured human THP-1 cells. Effect on human intestinal epithelial cell integrity was also assessed. Each cell type was incubated (±lipopolysaccharide [LPS]) with all-trans retinoic acid (ATRA), 13-cis-RA (isotretinoin) and 4-oxo-13-cis-RA. Cytokine analysis was performed by array analysis. Cultured human endothelial colorectal adenocarcinoma (Caco-2) cells were incubated with these retinoids and media analyzed for leakage by spectrofluorometric analysis. ATRA consistently and significantly inhibited LPS-induced release of the pro-inflammatory cytokines tumor necrosis factor, interleukin (IL)-6, macrophage inflammatory protein (MIP)-1 and MIP-1 . All retinoids tested stimulated release of the anti-inflammatory cytokines granulocyte-macrophage colony-stimulating factor and IL-10, and also monocyte chemotactic protein-1, vascular endothelial growth factor and eotaxin-1. Incubation with retinoids did not significantly alter the permeability of Caco-2 monolayers. Pre-treatment of each cell type with retinoids promoted an anti-inflammatory cytokine profile with only minimal effect on intestinal epithelial cell permeability; consistent with in vivo studies.

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The Effects of Vitamin A on Cells of Innate Immunity In Vitro

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Abbreviations used:

13-cis-RA = 13-cis-retinoic acid; 4-oxo-13-cis-RA = 4-oxo-13-cis-retinoic acid; ATRA = all-trans retinoic acid; Caco-2 = human endothelial colorectal adenocarcinoma; CD = Crohn's disease; DSS = dextran sodium sulphate; EDTA = ethylenediaminetetraacetic acid; FCS = fetal calf serum; FITC = fluorescein isothiocyanate; GI = gastrointestinal; GM-CSF = granulocyte-macrophage colony-stimulating factor; IBD = irritable bowel disease; ICAM-1 = intercellular adhesion molecule-1; IFN = interferon; IL = interleukin; IL-1RA, interleukin-1 receptor antagonist; *iv*DCs = *in vitro*-differentiated human dendritic cells; *iv*MACs = *in vitro*-

differentiated human macrophages; LPS = lipopolysaccharide; MCP = monocyte chemotactic protein; MIP = macrophage inflammatory protein; MMP = matrix metalloproteinase; TGF = transforming growth factor; THP = monocytic leukemia, TNBS = trinitrobenzene sulfonic acid; TNF = tumor necrosis factor; PBMCs = peripheral blood mononuclear cells; PBS = phosphate buffered saline; RH = relative humidity; UC = ulcerative colitis; VEGF = vascular endothelial growth factor

ABSTRACT

Retinoid treatment is suggested to promote development of inflammatory bowel disease, although preclinical studies are not supportive. We evaluated the effect of retinoids on cytokine response in *in vitro*-differentiated human dendritic cells (*iv*DCs) and macrophages (*iv*MACs) derived from healthy human donors and in cultured human THP-1 cells. Effect on human intestinal epithelial cell integrity was also assessed. Each cell type was incubated (± lipopolysaccharide [LPS]) with all-trans retinoic acid (ATRA), 13-cis-RA (isotretinoin) and 4-oxo-13-cis-RA. Cytokine analysis was performed by array analysis. Cultured human endothelial colorectal adenocarcinoma (Caco-2) cells were incubated with these retinoids and media analyzed for leakage by spectrofluorometric analysis. ATRA consistently and significantly inhibited LPS-induced release of the pro-inflammatory cytokines tumour necrosis factor, interleukin (IL)-6, macrophage inflammatory protein (MIP)-1α and MIP-1β. All retinoids tested stimulated release of the anti-inflammatory cytokines granulocyte-macrophage colony-stimulating factor and IL-10, and also monocyte chemotactic protein-1, vascular endothelial growth factor and eotaxin-1. Incubation with retinoids did not significantly alter the permeability of Caco-2 monolayers. Pre-treatment of each cell type with retinoids promoted an anti-inflammatory cytokine profile with only minimal effect on intestinal epithelial cell permeability; consistent with *in vivo* studies.

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Keywords: cytokines, inflammation in IBD, dendritic cells in IBD, macrophages in IBD.

1. Introduction

There is currently much debate as to whether vitamin A and associated retinoid derivatives are beneficial or harmful to the gastrointestinal (GI) tract, a situation primarily driven by clinical case reports claiming a putative causal relationship between retinoid treatment with 13-cis-retinoic acid (13-cis-RA, isotretinoin) and the occurrence of ulcerative colitis (UC) and Crohn's disease (CD), i.e. two forms of chronic inflammatory bowel disease (IBD) (Crockett et al., 2010; Reddy et al., 2006). Contrary to this, key basic research data do, in fact, support anti-inflammatory effects of retinoids on the GI tract (Bai et al., 2009; Iwata and Yokata, 2011). Nevertheless, the case for retinoids being beneficial or harmful to the GI tract has only infrequently been based on robust scientific evidence and, thus far, it has not been possible to confirm or refute a causative relationship (Crockett et al., 2009). Ideally, further prospective or well-designed retrospective pharmacoepidemiological studies are needed to definitively establish causality (Rogler et al., manuscript in preparation).

Understanding of the pathophysiology of IBD has markedly increased recently with a number of pre-disposing genetic risk factors identified for CD and (to a lesser extent) UC, along with a number of environmental triggers considered as potential key mediators of disease development (Rogler, 2011). Although more risk factors are expected to follow (Barrett et al., 2008; Latella et al. 2010; Nguyen et al., 2006), the role of many these in the pathophysiology of CD, for example, is unclear (Mathew, 2008) and, nevertheless, account for only a fraction of observed CD incidence (Torkamani et al., 2008). Key environmental triggers include dietary factors, food additives or drugs (Cosnes, 2010; Hou et al., 2011a; Hou et al., 2011b;

Järnerot et al., 1983; Katschinski et al., 1988; Martini and Brandes, 1976; Silkoff et al. 1980; Thornton et al., 1979), and cigarette smoking (Avidan et al., 2005; Cosnes et al., 2001; Cosnes et al., 1996; Kane et al., 2005) while psychological factors may influence disease course (Cámara et al., 2010; Danese et al., 2004; Levenstein, 2002). Significantly, the innate immune system and epithelial barrier function are critical to the pathophysiology of both UC and CD; thus, environmental triggers that influence the intestinal microbiota and modify intestinal barrier function would appear particularly relevant.

Recent studies in experimental models of IBD and in human colonic biopsy samples have shown retinoids to be potentially anti-inflammatory; for example, all-trans-retinoic acid (ATRA, tretinoin) and transforming growth factor (TGF)-β1 promoted differentiation of FOXP3+ regulatory T-cell subsets (Benson et al., 2007; Iwata and Yokota, 2011) and prevented differentiation of pro-inflammatory interleukin (IL)-17-secreting Th17 cells (Bai et al., 2009; Hundorfean et al., 2012; Nikoopour et al., 2008; Reifen et al., 2002). Notably, observations of lower levels of pro-inflammatory cytokines (tumor necrosis factor [TNF]-α, subsequently referred to as TNF, IL-1β, IL-17), increased levels of regulatory cytokines (IL-10, TGF-β), and a dose-dependent amelioration of 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis by ATRA were sufficiently strong for the authors to suggest ATRA as having therapeutic potential in IBD (Bai et al., 2009). Moreover, ATRA has been shown to be a critical regulator for barrier protection during mucosal injuries via up-regulation of tight-junction proteins and cyclo-oxygenase (Osanai et al., 2007). ATRA has also been shown to up-regulate the expression of gut-homing receptors e.g., integrin α4β7 and C-C chemokine receptor type 9 on T-cells in vitro, which, upon binding to mucosal cascular addresin cell adhesion molecule 1 and chemokine (C-C motif)

ligand 25, respectively, mediate the migration of Th17 cells and regulatory T cells to the gut mucosa (Iwata et al., 2004).

Nevertheless, data from studies in primary human cells and intestinal cell lines as to the effects of retinoids remain limited. In this in-vitro study we evaluated the effects of retinoid derivatives of vitamin A – ATRA (tretinoin, the major metabolic derivative of vitamin A), 13-cis-RA (isotretinoin) and 4-oxo-13-cis-retinoic acid (4-oxo-13-cis-RA, the primary stable metabolite of isotretinoin) – on lipopolysaccharide (LPS)-induced cytokine release from differentiated monocytic dendritic cells and macrophages, and from cultured human acute monocytic leukaemia (THP)-1 cells, and also their effects on human intestinal epithelial cell integrity. The effect of retinoids in *in vivo* animal models has been investigated also (data to be published separately).

2. Materials and methods

2.1. Retinoids

ATRA, 13-cis-RA and 4-oxo-13-cis-RA (RO22-6595, Roche, Switzerland) were dissolved in dimethylsulfoxide (40 mg/mL), diluted in phosphate buffered saline (PBS), and tested at final concentrations of 0.01–5.0 µg/mL.

2.2. Human donors

Peripheral blood from healthy donors (two males and five females, aged 25–43 years) was obtained after oral consent, and in accordance with the guidelines of the ethical committee of Canton Zurich.

2.3. Isolation of human peripheral blood monocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from 80 mL of peripheral blood according to the Ficoll® gradient centrifugation method. PBMCs were incubated with magnetic microbeads (130-091-153, Monocyte Isolation Kit II, Miltenyi Biotec, Bergisch Gladbach, Germany) in accordance with the manufacturer's protocol, and final isolation of monocytes was achieved using a magnetic cell sorter (AutoMACS, Miltenyi Biotec, Germany).

2.4. Generation of in vitro*-differentiated dendritic cells (*iv*DCs)*

PBMCs were differentiated into dendritic cells using an established protocol (Rogler et al., 1998); monocytes were cultivated in flasks for 1 week under optimal conditions (37°C, 5% CO_2, 95% relative humidity [RH]) with 5 ng/mL IL-4 and 50 ng/mL granulocyte-macrophage colony-stimulating factor (GM-CSF).

2.5. Generation of in vitro*-differentiated macrophages (*iv*MACs)*

As described above for *iv*DCs, peripheral blood monocytes were differentiated into macrophages based on the established protocol cited, with monocytes being cultivated in Teflon bags for 1 week under optimal conditions (37°C, 5% CO_2, 95% RH). Differentiated macrophages were detached from the Teflon bags by incubation at 4°C.

2.6. Cell culture (THP-1 and Caco-2 cells)

The monocytic/macrophage-like THP-1 cell line was cultivated in Roswell Park Memorial Institute medium containing 10% fetal calf serum (FCS), supplemented with penicillin (100 U/mL) and streptomycin (100 µg/mL) under standard conditions (37°C, 5% CO_2, 95% RH). Human epithelial colorectal adenocarcinoma (Caco-2) cells were grown in high glucose Dulbecco's Modified Eagle's Medium containing 10% FCS, supplemented with penicillin (100 U/mL) and streptomycin (100 µg/mL) under recommended conditions (37°C, 10% CO_2, 95% RH). For both cell lines, passaging was carried out according to the guidelines of the American Type Culture Collection (ATCC 2012).

2.7. Stimulation of cytokine response

The influence of retinoids on the LPS-induced cytokine response of *iv*DCs, *iv*MACs and THP-1 cells was evaluated in each cell type using the same experimental methodology. Primary cells were adjusted to a density of 1×10^6 cells/mL and plated onto 96-well plates (100 µL/well). THP-1 cells were incubated in six-well plates at a density of 7×10^5 cells/mL. Retinoids were added to the medium (0.01, 0.1, 1.0 and 5.0 µg/mL) for ivDCs, ivMACs and THP-1 cells, and pre-incubated

for 1 hour prior to stimulation with LPS (to a final concentration of 100 ng/ml) for a further 48 hours at 37°C.

Incubation medium was collected and processed for cytokine analysis (Rules-Based Medicine, Austin, Texas, USA) using Human Cytokine MAP A 1.0® array. Levels of IL-15 were below the detection limit of the assay and were excluded from the analysis.

For studies in ivDCs, cytokine response data shown are based on at least six (LPS-induced) and at least four (no LPS) independently performed experiments, each corresponding to a different donor. In ivMACs, these data (both LPS-induced and no LPS) were each based on at least four independently performed experiments, each corresponding to a different donor. Data shown for cytokine response in THP-1 cells are based on three independent experiments.

2.8. Intestinal epithelial cell permeability

To investigate a potential role of retinoid derivatives of vitamin A on intestinal permeability, the diffusion of fluorescently-labelled dextran was measured across Caco-2 monolayers pre-incubated with retinoid. Caco-2 cells were grown onto trans-well inserts of 0.4 µm pore size for 3 weeks to reach maximum confluency. Cells were subsequently pre-incubated with different concentrations of retinoids (0.01, 0.1, 1.0 and 5.0 µg/mL) for 48 hours. Caco-2 monolayers were washed once with PBS and fluorescein isothiocyanate (FITC)-labelled 10 kDA dextran (Sigma-Aldrich, St Louis, USA) and added to the apical chambers at a final concentration of 0.2 mg/mL. Ethylenediaminetetraacetic acid (EDTA) 0.1 mM was used in parallel as a positive control. Following overnight incubation, media from the basal chambers were collected and analysed for FITC-dextran leakage using spectrofluorometric analysis

(Biotek, Winooski, USA). Data are provided based on mean values from two independent representative experiments.

2.9. Statistical analysis

Based on a paired analysis of LPS-induced responses, statistical significance was determined using a one-way analysis of variance with Tukey's multi-comparison post-test using Graph Pad Prism 5 software (GraphPad Software, La Jolla, California, USA).

3. Results

3.1. Cytokine response

3.1.1. iv*DCs*

In the presence of LPS, ATRA significantly inhibited the LPS-induced release of pro-inflammatory cytokines such as TNF, IL-6, macrophage inflammatory protein (MIP)-1α and MIP-1β from *iv*DCs (Fig. 1); data were consistent across all retinoid concentrations tested (0.01, 0.1, 1.0 and 5.0 µg/mL) and, for clarity, only 1 µg/mL data are shown. Additionally, ATRA and its derivatives significantly stimulated the production of monocyte chemotactic protein (MCP)-1 and vascular endothelial growth factor (VEGF), and also the anti-inflammatory cytokine IL-10 (Fig. 1). Although incubation of *iv*DCs with retinoids affected the LPS-induced release of several other cytokine targets implicated in the inflammatory response, none of these changes were significant (Supplementary Fig. I).

In the absence of LPS, incubation with ATRA and 13-cis-RA each induced increases in GM-CSF, MCP-1 and VEGF from *iv*DCs, which were significant at the highest doses tested; a similar but non-significant trend being evident for 4-oxo-13-cis-RA (Fig. 2). There was little or no change in the cytokine response for IL-1α, IL-1 receptor antagonist (IL-1RA), IL-4, and IL-18. Although there was a tendency for the retinoids tested to induce the release of intercellular adhesion molecule-1 (ICAM-1), interferon (IFN)-γ, IL-1β, lymphotoxin-α, matrix metalloproteinase (MMP)-2 and stem cell factor, and to also inhibit the release of IL-10, IL-6, MIP-1α, MIP-1β and TNF, these changes were modest and in all cases not statistically significant (Supplementary Fig. II).

3.1.2. iv*MACs*

In the presence of LPS, similarly significant increases were seen in the release of MCP-1, eotaxin-1, and VEGF following incubation of *iv*MACs with each retinoid (Fig. 3, consistent responses were again evident across all retinoid concentrations and, for clarity, only 1 µg/mL data are shown). Additionally, significant reductions were seen in the LPS-induced release of the pro-inflammatory cytokines TNF, IL-6, MIP-1α and MIP-1β (Fig. 3), i.e. responses consistent with those seen for *iv*DCs. Incubation of *iv*MACs with retinoids also tended to promote increased LPS-induced release of IL-8, IL-10, IL-1β and IL-1RA and reduction in the release of IL-1α, but these changes were not statistically significant (Supplementary Fig. III). Moreover, no changes were evident in the responses for ICAM-1, IL-18, and MMP-3.

In the absence of LPS, comparable responses to those observed for *iv*DCs were seen in that the retinoids tested induced the release of MCP-1, eotaxin-1, IL-8 and VEGF; for eotaxin-1 and VEGF, responses appeared dose dependent (albeit non-significant) for all retinoids tested (Fig. 4). There was little or no change in the cytokine response for ICAM-1, IL-1α, IL-1β, and IL-6. Although there was a tendency for the retinoids tested to induce the release of IL-10, IL-18 and MIP-1α as well as inhibit the release of pro-inflammatory IFN-γ, IL-1RA, MIP-1β, MMP-3 and TNF, these changes were modest and in all cases not statistically significant (Supplementary Fig. IV).

3.1.3. THP-1 cells

The effects of retinoids on LPS-induced cytokine response from THP-1 cells were generally similar to those observed for both *iv*DCs and *iv*MACs. Pre-incubation of THP-1 cells with retinoids resulted in reduced LPS-induced release of IL-6 and

increased release of IL-8 and to a minor extent -MCP-1; these responses were evident for each of the retinoids tested (Fig. 5) and generally consistent with responses seen in *iv*DCs and *iv*MACs (Figs. 1–4).

3.2. Intestinal permeability

Incubation of human Caco-2 cells with different concentrations each of ATRA, isotretinoin and 4-oxo-cis-RA resulted in no significant change in permeability of Caco-2 monolayers at all doses tested (Supplementary Fig. V). FITC-labeled dextran was observed to be translocated effectively in EDTA-treated monolayers, a finding consistent with the known potent adverse effect of this compound on tight-junction integrity (Tomita et al., 1994).

4. Discussion

Retinoid treatment has recently been suggested to play a pathophysiological role in the development of chronic IBD, a contention based essentially on several case reports (Crockett et al., 2009; Shale et al., 2009). However, key basic research data appear to contradict this in showing retinoids to be mainly associated with anti-inflammatory activity (Bai et al., 2009; Straus and Glass, 2007) and, for example, substitution of vitamin A in a TNBS rat model of colitis was found to ameliorate colitis according to histological scores and weight curves (Bai et al., 2009; Reifen et al., 2002). While the molecular effects of vitamin A and its retinoid derivatives are well understood based on studies in multiple in-vitro settings (Amann et al., 2011; Delacroix et al., 2010; Li et al., 2006; Norris et al., 1987; Wada et al., 2009), the data available from intestinal cell lines and primary human cells are generally limited; a situation that has led to speculation recently as to whether retinoids are harmful or beneficial to the GI tract (Crockett et al., 2010; Reddy et al., 2006), and suggested need for further study (Rogler et al., 2012, unpublished observation, manuscript in preparation).

The findings from this *in vitro* study, designed to evaluate the effect of retinoids on cytokine release and suppression, and GI integrity in various human immune cell types, clearly demonstrate that pre-treatment of *iv*DCs, *iv*MACs and cultured human THP-1 cells with ATRA, or the derivatives tested, promotes an anti-inflammatory pattern of cytokine release with little or no change in epithelial cell line integrity. This specifically relates to significant inhibition of LPS-induced release of pro-inflammatory cytokines such as TNF and IL-6, and also of MIP-1α and MIP-1β. These observations, and also the fact that all retinoids tested stimulated the release

of the anti-inflammatory cytokine IL-10 from *iv*MACs and *iv*DCs, collectively confirm that retinoids promote a pattern of cytokine release that is more anti-inflammatory than pro-inflammatory. Such a pattern is, in fact, consistent with recent *in vitro* and *in vivo* studies. For example, retinoids such as ATRA play a crucial role in the differentiation of T-cells by inducing the differentiation of gut-homing FOXP3+ regulatory T-cells and preventing the differentiation of pro-inflammatory IL-17-secreting Th17 cells (including in human colonic biopsies) (Bai et al., 2009; Iwata and Yokota, 2011); they also promote the homing of Th17 cells and regulatory T cells to the GI mucosa and stimulation of antigen-presenting cells to secrete IL-10 (Benson et al., 2007; Crockett et al., 2009; Hundorfean et al., 2012; Nikoopour et al., 2008). Additionally, ATRA treatment has been shown to reduce inflammation, mucosal damage and myeloperoxidase activity in the mouse TNBS colitis model. In this study, lamina propria mononuclear cells from ATRA-treated animals were reported to produce lower levels of pro-inflammatory TNF, IL-1β, and IL-17 and release more regulatory cytokines (IL-10 and TGF-β) (Bai et al., 2009).

In the absence of LPS, incubation with each of the retinoids tested was similarly associated with little, or no, effect on the release of inflammatory mediators from all cell types; an effect that was observed over a broad range of retinoid concentrations (0.01, 0.1, 1.0 and 5 µg/mL). Under these conditions, the retinoids tested induced the release of eotaxin-1, MCP-1 and IL-8, i.e. chemokine targets involved in the migration of immune cells, and also GM-CSF and VEGF, from *iv*DCs and *iv*MACs. Perhaps most notable is the markedly increased release of GM-CSF, MCP-1 and VEGF in response to retinoids alone. There is now strong evidence, including studies both in GM-CSF knockout mice (Xu et al., 2008) and in human subjects (Goldstein et al., 2011), to support a key role of GM-CSF in activating and

maintaining intestinal innate immune barrier function. Collectively, such findings have fostered the emergence of CSFs as a potential tool for the treatment of IBD (Barahona-Garrido and Yamamoto-Furusho, 2008) and, in fact, recent controlled clinical trials have shown treatment with recombinant human GM-CSF to decrease disease severity and improve the quality of life of patients with active CD (Goldstein et al., 2011; Korzenik et al., 2005). It follows, therefore, that the retinoid-induced release of GM-CSF reported here, as distinct from LPS-induced responses, would provide potential benefit to the GI environment, particularly in pathological states such as IBD.

A similar view could be taken regarding the observed changes in MCP-1. This key target, together with IL-10, is crucial for the regulation of immune responses against commensal bacteria by intestinal macrophages (Takada et al., 2010) and has been shown also to exert a beneficial effect on dextran sodium sulphate (DSS)-induced colitis in mice (Maharshak et al., 2010). Thus, as for GM-CSF, the retinoid-induced release of MCP-1 seen in this study, both in the presence and absence of LPS, may similarly preclude a beneficial effect of this chemokine in steady-state gut homeostasis. In contrast, however, overexpression of VEGF-A has been shown to be associated with deterioration in disease status in mice with DSS-induced colitis, levels correlating with increased angiogenesis and leukocyte adhesion in the intestine (Scaldaferri et al., 2009), while increased levels of VEGF are usually observed in human subjects with IBD (Tsiolakidou et al., 2008). The release of VEGF might, therefore, be expected to convey potentially negative effects on intestinal immunology. To counterbalance this argument, VEGF has also been observed to inhibit the apoptosis of intestinal epithelial cells – thus preventing bacterial translocation across ileal mucosa (Nakajima et al., 2007) – while levels of

VEGF expression are reported as not being associated with disease activity in patients with IBD (Alkim et al. 2012). Nevertheless, until more data become available relating to the effect of VEGF on maintenance of gut homeostasis, it is perhaps prudent that caution is exercised in assessing the overall effect of this cytokine target on the intestinal milieu.

All retinoids tested were also found to have little or no adverse effect on the permeability of Caco-2 monolayers. This was also evident at all doses tested and is in apparent conflict with a relatively early *in vitro* study, which showed that the permeability of the Caco-2 monolayer, as measured by transepithelial electric resistance and [^{3}H]-mannitol flux, was enhanced by ATRA. Given the known association between vitamin A deficiency and impairment in intestinal integrity, the authors considered this surprising and attributed increased permeability to an unknown mechanism(s) and not altered tight-junction protein expression (Baltes et al., 2004). The findings with regard to permeability are, however, consistent with reported protective effects of retinoids for the GI mucosa. For example, gastric mucosal protection (against indomethacin treatment) was seen in healthy persons and in patients with gastric ulcer and duodenal ulcer without any inhibition of gastric acid secretion (Mózsik et al., 2001), while increased mucin production in the presence of retinoids was considered to contradict any putative drying effect of retinoid analogues on the intestinal epithelium as a causal contributor of IBD (Gray et al., 2001; Tan et al., 2007).

In summary, these *in vitro* findings confirm that retinoid derivatives of vitamin A provoke an LPS-induced cytokine response from human immune cells consistent with an anti-inflammatory pattern and with little or no adverse effect on intestinal

epithelial permeability. As such, these studies do not support retinoids as presenting a metabolic milieu dangerous to the GI tract. These findings are consistent with studies in *in vivo* animal models of colitis (to be published separately).

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Fig. 1. The influence of retinoids on LPS-induced release of inflammatory targets from *iv*DCs

Cell-treated supernatants were analyzed for different cytokines and chemokines after incubation with retinoids (1 µg/mL) and stimulation with LPS (100 ng/mL). Bars represent mean +SEM values from at least six independent experiments corresponding to different donors.

*$P < 0.05$; ***$P < 0.001$

Fig. 2. The influence of retinoids on the release of inflammatory targets from *iv*DCs

Cell-treated supernatants were analyzed for different cytokines and chemokines after incubation with different concentrations of retinoids (0.01, 0.1, 1.0 and 5.0 µg/mL). Bars represent mean +SEM values from at least four independent experiments corresponding to different donors.

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

Fig. 3. The influence of retinoids on LPS-induced release of inflammatory targets from *iv*MACs

Cell-treated supernatants were analyzed for different cytokines and chemokines after incubation with retinoids (1 µg/mL) and stimulation with LPS (100 ng/mL). Bars represent mean +SEM values from at least four independent experiments corresponding to different donors.

$^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

Fig. 4. The influence of retinoids on the release of inflammatory targets from *iv*MACs

Cell-treated supernatants were analyzed for different cytokines and chemokines after incubation with different concentrations of retinoids (0.01, 0.1, 1.0 and 5.0 µg/mL). Bars represent mean +SEM values from at least four independent experiments corresponding to different donors.

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

Fig. 5. The influence of retinoids on the release of inflammatory targets from THP-1 cells

Cell-treated supernatants were analyzed for different cytokines and chemokines after incubation with different concentrations of retinoids (0.01, 0.1, 1.0 and 5.0 µg/mL) either in the absence (light bars) or presence (dark bars) of LPS (1 µg/mL). Bars indicate mean values from three independent experiments.